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Peculiarities of two different IAA binding sites functioning in kidney bean hypocotyl cell plasmalemma

M. Jodinskienė,

N. Anisimovienė

Institute of Botany,
Laboratory of Plant Physiology,
Žaliųjų Ežerų 49, LT-08406 Vilnius,
Lithuania
E-mail: mildajod@yahoo.com

Peculiarities of the interaction of phytohormone indole-3-acetic acid (IAA or auxin) and auxin-binding proteins (ABP) in the plasmalemma of kidney bean (dicot plant) hypocotyl cells according to different physiological responses to IAA have been studied.

Two different IAA binding sites (optimal pH 5.5 and 7.5) were discovered in the plasmalemma of cells responding to IAA by elongation. The first site is characterized as IAA-receptor mediating cell response to IAA by elongation. It has a structure of the IAA binding site – /-H-R-H-S-C-E-/. This site is not functioning in hypocotyl cells not responding to IAA by elongation.

The activity of the second site (optimal pH 7.5) is dependent on IAA transport. Basing on the characteristics of this site, the following suggestions may be made: one residue of histidine and amino acid(s) containing the -SH- group are functioning in this site; the IAA influx inhibitor 3-chloro-4-hydroxyphenylacetic acid (CHPAA) is capable of a very active competition with IAA in the binding site: it reduces IAA–ABP interaction by 92%.

Our results confirmed that several different ABP may be functioning in the cell and that for each separate response an individual receptor may be required.

Key words: indole-3-acetic acid (IAA), auxin-binding protein (ABP), auxin influx inhibitors, auxin efflux inhibitors

INTRODUCTION

The action of IAA undoubtedly starts at the moment when the molecule of this phytohormone interacts with a specific ABP–IAA-receptor which after binding with a phytohormone induces modification of the expression of several gene families (*SAURs, Aux/IAA, GH$_3$*) , synthesis of new polypeptides [1–3].

Over the last three decades, attention has been concentrated on elucidation of IAA binding sites and characterization of ABPs functioning in monocot [3–6] and dicot [7–9] plant cells growing by elongation. Several IAA binding sites (differing in their pH, molecular weight of ABP, amino acid composition, K_D, number of IAA binding sites) and the formation of IAA–receptor complexes in the plasmalemma and cytosol of these plants have been disclosed [3, 5, 10, 11]. Recently, IAA binding sites have been formed in the nucleus [12].

The molecular mechanism of IAA action – the biochemical events leading to one or another physiological response to IAA – are still under investigation. There are no answers how many ABP–IAA receptors are functioning in the cell, whether all responses to IAA are realized through one universal receptor, on what rank of the ABP structure the specificity of ABP–IAA interaction depends, how the characteristics of IAA–ABP complex that participate or/and determine particular physiological response differ?

The aim of our investigation was to elucidate IAA–ABP interaction peculiarities in the plasmalemma of kidney bean (dicot plant) cells differently responding to IAA.

MATERIALS AND METHODS

4–5-day-old etiolated kidney bean (*Phaseolus vulgaris* L.) hypocotyl segments, responding and not responding to IAA by elongation growth, were used as the test object. Optimal IAA concentration, IAA transport rate, and differently elongating hypocotyl zones were determined experimentally using 1% agar blocks-donors containing IAA at various concentrations (50, 10, 5, 1 μM) [13, 14]. Attention was focused on the intensively elongating hypocotyl zone; experiments were started in the basal – mature [15] weakly elongating zone.

Plasmalemma vesicle fraction was isolated by differential centrifugation and purified in sucrose step gradient [6, 8, 10, 16]. The plasmalemma fraction (1.13–1.17 d sucrose interface) was characterized according to K^+Mg^{2+}-ATPase activity considering the effect of specific inhibitors, contamination with other subcellular compartments. Plasmalemma proteins were solubilised by the Triton X-100 non-ionic detergent.

The chelato-affinity chromatography method (Sepharose-type matrix with an immobilized specific ligand – iminodiacetic acid (26 μM/ml), chelator – Cu^{2+} ions, competing ligand – imi-

dazole) was applied for isolation of proteins interacting with IAA [8, 11]. ABP were purified employing standard procedures – multistage gel-filtration column chromatography, dialysis [8], native PAGE. Molecular weight (kDa) was determined by localization of molecular mass standards [17]. Protein content in all stages of experiments was determined by the method of Bradford [18]; bovine serum albumin (BSA) was used as a standard.

Formation of specifically bound IAA–ABP complexes and their characteristics were evaluated by formation of IAA–ABP complexes [4, 8, 16, 19]. ^{14}C-IAA concentration was 0.5 μM, ^{12}C-IAA 100 μM, ^{14}C-IAA specific activity 2120 GBq/mol. Radioactivity was measured in Bray's system with a Beckman LS 1801 liquid scintillation counter.

Formation of kidney bean IAA–plasma membrane protein complexes was examined in differently elongating hypocotyl zones (treated and not treated with 10 μM IAA for 1 h). Specific compounds modifying -SH- groups in proteins – N-ethylmaleimide (100 μM), iodoacetic acid (100 μM), DTT (100 μM) and $K_3Fe(CN)_6$ (500 μM) – were used for the elucidation of amino acids present in the IAA binding site and functioning in IAA–ABP interaction [4, 19].

IAA efflux inhibitors 2,3,5'-triiodobenzoic acid (TIBA), 1-N-naphtylphthalamic acid (NPA) and IAA influx inhibitors 1-naphthoxyacetic acid (1-NOA), 3-chloro-4-hydroxyphenylacetic acid (CHPAA) were used (100 μM) for evaluation of their ability to compete with IAA for binding sites [14, 19, 20]. The arithmetical mean from 3–5 experiments and their standard errors are presented. Only differences significant at $p \geq 0.95$ were taken into consideration.

RESULTS AND DISCUSSION

Formation of IAA–protein complexes was examined in the apical hypocotyl zone elongating most intensively (2 cm long, underlying 5 mm down to cotyledons) and in the basal zone (Fig. 1 A). Two different IAA binding sites (optimal pH 5.5 and 7.5) functioning in the plasmalemma of cells responding to IAA by elongation were discovered [8, 13]. The first binding site (optimal pH 5.5) has the amino acid sequence /-His-Arg-His-Ser-Cys-Glu-/ and the IAA carboxyl group binding cluster /-His-Arg-His-/, 26 kDa; it is a monomer; Zn^{2+} is localized in its active centre [8, 11]. This binding site is the same as or analogous to that of the ABP1–IAA binding protein functioning in coleoptile plasmalemma of maize (monocot plant) [3, 5, 9]. This structure of the IAA binding site is characteristic of the IAA receptor mediating cell response to IAA by elongation both in monocot and dicot plants [9, 21, 22].

The second ABP (29–45 kDa, pH 7.5, n – 40.00 ± 1.77 pmol/1g f. w., K_D – 5.86 × 0.1 μM) is functioning in the plasmalemma isolated from the cells responding to IAA by elongation [13, 14, 22]. So far, the functioning of IAA binding site (optimal pH 7.0–7.5) has been revealed in maize plasmalemma [23] and wheat coleoptile cells [6, 10]. The IAA–ABP complex (optimal pH 7.2) forming in the plasmalemma of elongating wheat coleoptile cells increases I and II RNA polymerase activity in the nucleus [6, 10].

While analyzing the peculiarities of IAA–plasmalemma protein binding and characteristics of the formed IAA–ABP (totally and specifically bound IAA per protein unit, their specificity, %) in the hypocotyl zone not responding to IAA by elongation, it has been determined that the site at pH 5.5 is not functioning and no IAA–ABP complex with characteristics typical of IAA–protein receptor mediating cell responses to IAA by elongation is formed.

According to preliminary results, formation of IAA–ABP complex at pH 7.5 in the basal zone of hypocotyls is weak – 2.24 ± 0.24% (110.00 ± 5.77 cpm/1mg protein), yet IAA pretreatment (10 μM) increases formation of specific IAA–ABP complexes – ^{14}C-IAA bound specifically 1124.67 ± 52.79 cpm/1mg protein, specificity 27.66 ± 1.36% (Fig.1 B). Consequently, the IAA–ABP complexes formed at pH 7.5 are not so closely related to elongation growth as the IAA–ABP complex formed at pH 5.5. It may be suggested that functioning of this site in mature hypocotyl cells is related to IAA transport.

Having in mind that not only ABP IAA-receptors but also IAA-transporters and IAA-enzyme complexes are functioning in the cell [23, 24] and that the structure of IAA binding site significantly influences the function of the complexes [9, 11, 21],

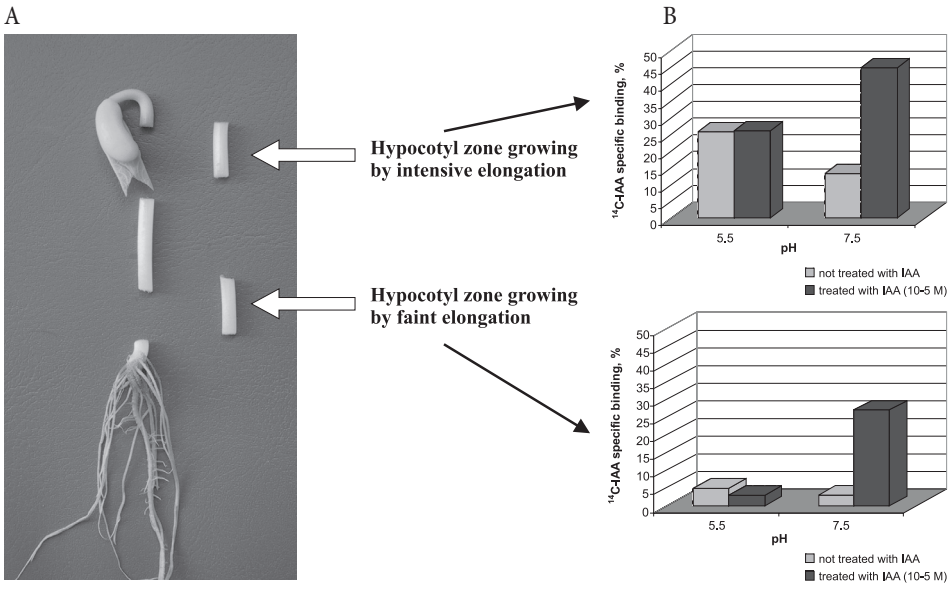

Fig. 1. Zones of kidney bean hypocotyls responding to IAA by unequal intensity of growth (A) and specific ^{14}C-IAA binding in plasmalemma vesicles isolated from differently elongating hypocotyl zones (B)

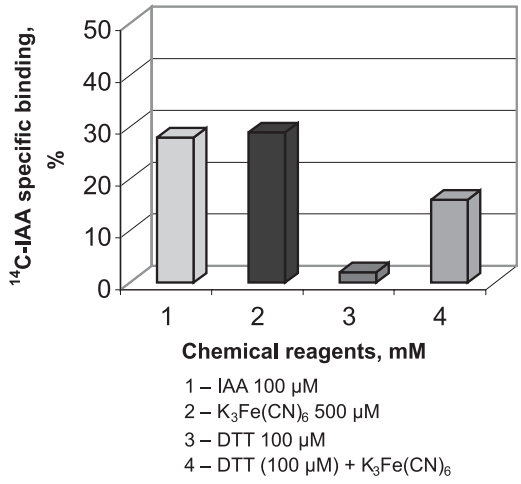

Fig. 2. Dependence of IAA–ABP complex formation in plasmalemma proteins (pH 7.5) on -SH- group modifying reagents

analysis of the site structure of the ABP–IAA complex formed at pH 7.5 in cells not responding to IAA by elongation was started.

According to the results of chelato-affinity chromatography [8], one /-His-/ residue is localized in this protein binding site, i. e. elution of immobilized proteins is achieved only by a competing ligand – imidazole. Analysis of the effects of sulfhydryl group modifying reagents (N-ethylmaleimide, iodoacetic acid, DTT) on the formation of specific IAA–ABP complexes in kidney bean plasmalemma from the weakly elongating hypocotyl zone at pH 7.5 has shown that N-ethylmaleimide and iodoacetic acid has no significant influence on the specific IAA binding. When introduced to the reaction mixture, DTT significantly reduced specific IAA binding from 28.00 ± 1.46% (165.40 ± 8.61 cpm/1 mg protein) to almost none. Similar results were obtained in both plasmalemma vesicles and purified protein specimens. It is known that the participation of -SH- group containing amino acid in IAA binding site may be proved by impairing a negative DTT influence by subsequent treatment of membrane particles with ferricyanide $K_3Fe(CN)_6$ [19]. In our experiments this feature was confirmed also. When $K_3Fe(CN)_6$, was introduced separately, it had no influence on specific IAA binding, yet, when introduced additionally after DTT (Fig. 2), it restored this impact, at least partially (up to 16.82 ± 1.55%). The inhibitory DTT impact was also demonstrated while analysing protein preparations purified in several stages. DTT reduced specific IAA binding from 22.83 ± 0.94% (1143.37 ± 47.71 cpm/1 mg protein) to 0 %, and $K_3Fe(CN)_6$ restored it up to 13.86 ± 1.41% (860 ± 57.82 cpm/1 mg protein) in the case when purified proteins were extracted from PAGE. These results show the possible presence of amino acid containing -SH- groups located at the IAA–ABP binding site and could be essential for IAA binding activity. It may also be a cysteine as in the case of the first IAA–ABP complex (formed at pH 5.5 in hypocotyls responding by elongation).

Four IAA transport inhibitors have been tested to reveal their possibility to compete for IAA binding site (optimal pH 7.5) functioning in kidney bean hypocotyl cells (Fig. 3 A, B). Both IAA efflux inhibitors have blocked specific ^{14}C-IAA binding: TIBA up to 10.62 ± 2.26% (120.68 ± 25.67 cpm/1 mg protein) and NPA to 16.5 ± 3.22% (142.45 ± 10.11 cpm/1 mg protein) respectively, versus 37.24 ± 1.92% (specific binding

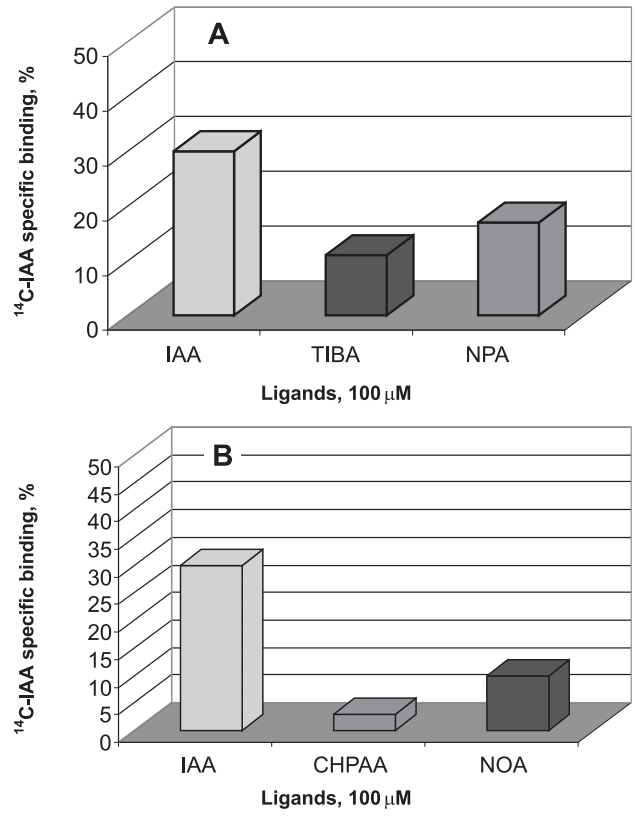

Fig. 3. Specificity of IAA efflux (A) and influx (B) inhibitors when competing for ^{14}C-IAA binding sites at pH 7.5

297.25 ± 15.37 cpm/1 mg protein) in control (Fig. 3 A). IAA influx inhibitors 1-NOA and CHPAA have also blocked specific ^{14}C-IAA binding by 10.64 ± 1.49% (84.64 ± 11.89 cpm/1 mg protein) and 2.92 ± 0.51% (39.09 ± 6.68 cpm/1mg protein), respectively. These results, especially the fact that the auxin influx inhibitor CHPAA blocks even up to 92% of specific binding, imply that IAA binding site functioning at pH 7.5 may be associated with IAA transport.

Contrary to the investigations on ABP–IAA receptors acting in the plasmalemma of cells responding to IAA by elongation [4, 6, 22, 25], the question on IAA receptors and IAA–ABP interaction peculiarities in relation to their response to IAA by division, developmental processes are poorly investigated [26]. The peculiarities of endogenous auxin – IAA in the hypocotyl zone not responding to IAA by elongation – have not been investigated so far. However, in one publication, the action of synthetic auxin 2,4 D in elongating and mature soybean hypocotyl cells was compared [15]. According to Key [15], auxin affects the radial expansion of cells and enhances the expression of different specific mRNR in the mature hypocotyl zone.

Thus, the obtained results [14, 22, 27] show that several different ABP may be functioning in different compartments of dicot plant cells; they are also in agreement with the data of other investigators [12, 15, 26], showing that an individual (specific) receptor is required for each response of the cell.

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DVIEJŲ SKIRTINGŲ IAR SAITŲ, FUNKCIONUOJANČIŲ PUPELIŲ HIPOKOTILIŲ PLAZMOLEMOJE, YPATYBĖS

Santrauka

Tiriami fitohormono indolil-3-acto rūgšties (IAR arba auksino) ir auksiną prisijungiančių baltymų (ASB) sąveikos ypatumai pupelių (dviskiltis augalas) hipokotilių ląstelėse priklausomai nuo atsako į IAR poveikį.

Ląstelių, atsakančių į IAR poveikį tįstamuoju augimu, plazmolemoje aptikti du skirtingi IAR prijungimo saitai (pH 5,5 ir pH 7,5). Pirmasis iš jų yra charakterizuotas kaip IAR receptorius, nulemiantis ląstelės atsaką į IAR poveikį tįstamuoju augimu. Šiam IAR prijungimo saitui būdinga amino /-H-R-S-C-E-/ rūgščių seka. Pupelių hipokotilių ląstelių plazmolemoje, nereaguojančioje į IAR poveikį tįstamuoju augimu, šis saitas nefunkcionuoja.

Antrojo saito (optimalus pH 7,5) aktyvumas priklauso nuo IAR transporto. Saito charakterizavimo rezultatai leidžia daryti šias prielaidas: šiame saite funkcionuoja viena histidino rūgšties liekana ir -SH-grupę turinti aminorūgštis; IAR įnešantis inhibitorius – 3-chloro-4-hidroksifenilacto rūgštis (CHPAR) – labai aktyviai konkuruoja dėl IAR prijungimo saito – sumažina auksino ir baltymo sąveiką iki 92%.

Raktažodžiai: indolil-3-acto rūgštis (IAR), auksiną prisijungiantis baltymas (ASB), auksiną „įnešantys“ inhibitoriai, auksiną „išnešantys“ inhibitoriai